RESEARCH ARTICLE

A_1 Adenosine Receptors Accumulate in Neurodegenerative Structures in Alzheimer's Disease and Mediate Both Amyloid Precursor Protein Processing and Tau Phosphorylation and Translocation

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Immunostaining of adenosine receptors in the hippocampus and cerebral cortex from necropsies of Alzheimer's disease (AD) patients shows that there is a change in the pattern of expression and a redistribution of receptors in these brain areas when compared with samples from controls. Adenosine A_1 receptor (A_1R) immunoreactivity was found in degenerating neurons with neurofibrillary tangles and in dystrophic neurites of senile plaques. A high degree of colocalization for A_1R and βA4 amyloid in senile plaques and for A_1R and tau in neurons with tau deposition, but without tangles, was seen. Additionally, adenosine A_{2A} receptors, located mainly in striatal neurons in controls, appeared in glial cells in the hippocampus and cerebral cortex of patients. On comparing similar samples from controls and patients, no significant change was evident for metabotropic glutamate receptors. In the human neuroblastoma SH-SY5Y cell line, agonists for A_1R led to a dose-dependent increase in the production of soluble forms of amyloid precursor protein in a process mediated by PKC. A_1R agonist induced p21 Ras activation and ERK1/2 phosphorylation. Furthermore, activation of A_1R led to and ERK-dependent increase of tau phosphorylation and translocation towards the cytoskeleton. These results indicate that adenosine receptors are potential targets for AD.

Introduction

Alzheimer's disease (AD) is a neurodegenerative condition with a high incidence over the age of 85 years. Thus, this disease has a great socio-economic impact in industrialized societies where the life expectancy is longer. Although the triggering factors are not known, it is known that progressive synaptic and neuronal loss occurs. This degeneration is more prominent in the temporal and hippocampal structures (15, 40). Although there are pharmacological approaches to slow down memory loss and cognitive impairment, the discovery of novel therapeutic targets is essential for the struggle against this disease

The 2 defining pathological hallmarks of AD are neurofibrillary tangles and amyloid plaques. Contradictory hypotheses exist as to whether tangles and plaques are the necessary and sufficient condition for the development of AD and as to the putative primacy of one lesion over the other (32). Tangles consist of microtubule-associated tau protein, normally expressed in axons, but in AD this protein becomes highly phosphorylated and aggregates into abnormal filaments in the cell body, thereby impairing the normal role of tau in tubulin polymerization and stabilization (30). Different kinases are able to phosphorylate tau in vitro (2, 22), including mitogen-activated protein kinases (MAPK). MAPK/ERKs phosphorylate tau and neurofilament proteins (28, 41). Moreover, certain MAPKs related with tau phosphorylation are overexpressed in AD brain (11, 12, 43, 44) and in other taupathies (11, 12). AD is also characterized by amyloid plaque deposition. Ab1-42 peptide is the main peptide component of plaques and is organized in thick fibrils intermixed with non-fibrillar forms of the peptide. Plaques also contain degenerating axons and dendrites, and these are surrounded by some microglia and reactive astrocytes (7). The Aβ1-42 peptide is derived from the membrane-bound amyloid precursor protein (APP). In nonpathological conditions

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cellular turnover of APP is accompanied by the release in the medium of the soluble ectodomain of the protein (sAPP), detected not only in the conditioned medium of transfected cells but also in the plasma and cerebrospinal fluid. Proteolisis occurs primarily by cleavage between amino acids 16 and 17 inside of the Aβ sequence, precluding Aβ1-42 formation. It is believed that α-secretases are responsible for this proteolisis generating a nonamyloidogenic peptide (42). The α-secretase can be stimulated by protein kinase C (23). An alternative cleavage is catalyzed by β-secretases giving rise to a soluble protein β-sAPP and amyloidogenic peptides Aβ 1-40, Aβ 1-42 (7, 38). An imbalance between Aβ production and Aβ clearance is implicated in AD pathogenesis (19)

It has been suggested that G-protein coupled receptor function may be compromised in AD (14), but the role of G protein coupled receptors in tau phosphorylation and in normal APP processing is unclear. Adenosine is a neuromodulator acting through 4 different subtypes of G-protein coupled specific receptors (A_1, A_{2A}, A_{2B} and A_3). These receptors are coupled to many signal transduction pathways, eg, stimulation (A_{2A} and A_{2B}) or inhibition (A_1 and A_3) of adenylate cyclase, stimulation of PLC (A_1 and A_{2B}). The 4 subtypes are able to induce ERK1/2 phosphorylation (25, 37). The receptors are distributed differently in various mammalian brains (10). An immunohistochemical study in human brain has shown that pyramidal cells are strongly labeled with anti-A_1R antibodies. In the hippocampus the strongest immunoreactivity is found in CA3 and in the hilus, whereas no clear staining is found in CA1, 2 regions (36). There is only one study that, to our knowledge, attempts to measure variations of A_1R levels in AD brain samples. This autoradiographic study reports the presence of A_1R in hippocampal regions and a diminution in the intensity of the radioligand binding in CA1 regions in patients (6).

The aim of this study was to map adenosine receptors in AD (hippocampus and frontal cortex) and to study their possible involvement in APP processing and tau phosphorylation. The results indicate that there is a preferential localization of these receptors in neurodegenerative structures. Moreover, activation of A_1R in a human neuroblastoma cell line regulates the production of soluble APP and leads to activation of $p21^{ras}$ and of ERK-mediated tau phosphorylation and translocation. A_1R can then be considered a potential target in the therapy for AD.

Materials and Methods

Human samples. Samples from hippocampus, neighboring entorhinal and temporal isocortex, and frontal cortex were obtained from 8 patients with sporadic, late-onset AD stage VI of tau pathology and stage C of βA4 amyloid deposition of Braak and Braak (1). The cases were 4 men and 4 women, and the mean age was 72.3 years. Six age-matched with no neurological disease were used as controls. The delay between death and tissue processing was between 2 and 7 hours in control and diseased brains. A complete neuropathological examination was carried out in every case (diseased cases and controls) in formalin-fixed tissue for no less than 3 weeks; the tissue was then embedded in paraffin. Dewaxed sections, 7-μm thick, were stained with haematoxylin and eosin, luxol fast blue-Klüver Barrera, methenamine silver (PAM) and Gallyas, or processed for immunohistochemistry following the avidin-biotin-peroxidase method (ABC kit, Vectastain, Vector). Antibodies to phosphorylated neurofilaments of 170 kD or 200 kD (clones BF10 and RT97, Boehringer-Mannheim) were used at dilutions of 1:100 and 1:50, respectively. Antibodies to pan-tau (Sigma) were used at a dilution of 1:10. Antibodies to glial fibrillary acidic protein (GFAP, Dako, Dakopats), βA4-amyloid (Boehringer-Mannheim), and ubiquitin (Dako) were used at dilutions of 1:250, 1:5, and 1:200, respectively. Control brains showed no abnormalities excepting a few βA4-amyloid plaques in the hippocampus in 2 cases.

For RNA extraction, gel electrophoresis and Western blotting, fresh samples were rapidly frozen on dry ice and stored at -80ºC until use

Immunohistochemistry. Immunohistochemistry was carried out following the streptavidin-biotin-peroxidase (LSAB) method (DAKO LSAB2 System Peroxidase; DAKO, Ely, United Kingdom). First, dewaxed sections were boiled in citrate buffer for 25 minutes and stored for 2 hours at room temperature. After blocking endogenous peroxidase with 0.3% hydrogen peroxide and 10% methanol for 15 minutes, sections were incubated with 3% normal horse serum for 2 hours and then incubated at 4ºC overnight with one of the primary antibodies. The anti-adenosine A_1 receptor rabbit polyclonal antibody (PC21, 5, 16), anti-adenosine A_{2A} receptor rabbit polyclonal antibody (VC21, 13, 35) and anti-metabotropic glutamate receptor 1 rabbit polyclonal antibody (F1, 13) were used at final concentrations of 53 μg/ml, 90 μg/ml and 20 μg/ml, respectively. To assess the specificity of the antibodies against adenosine A_1 or

A_{2A} receptors, some tissue sections were incubated with the antibody in the presence of ten times the quantity of peptide which recognizes the antibody. Sections were incubated with biotinylated anti-rabbit IgG secondary antibody for 10 minutes, followed by LSAB for 10 minutes at room temperature. The peroxidase reaction was visualized with 0.05% 3-3′-diaminobenzidine (Sigma-Aldrich) and 0.01% hydrogen peroxide. Double-labelling immunohistochemistry was carried out following a 2-step protocol. Sections were first incubated with antibodies against adenosine A_1 or A_{2A} receptors, and as before, the immunoreaction was visualized with diaminobenzidine and hydrogen peroxide. After washing, sections were incubated overnight with anti-tau antibody (Sigma-Aldrich, 1/1000) or anti-βA4 amyloid (DAKO, 1/50). Tissue sections processed for βA4 amyloid were pretreated with 96% formic acid for 5 minutes. Then sections were incubated with biotinylated anti-rabbit IgG secondary antibody for 10 minutes, followed by LSAB for 10 minutes at room temperature. Tomato lectin histochemistry was carried out using biotinylated lectin from *Lycopersicon esculentum* (Sigma-Aldrich) at a dilution of 1/100, followed by the avidin-biotin-peroxidase technique (ABC kit, Vector laboratories).

The immunoreaction was visualized with 0.01% benzidine hydrochloride, 0.025% sodium nitroferricyanide in 0.01M sodium phosphate buffer (pH 6.0), and 0.005% hydrogen peroxide.

Cell culture. Human neuroblastoma SH-SY5Y cells were cultured at 37ºC in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2mM L-glutamine and 1% (v/v) sodium pyruvate in a humidified atmosphere of 95% air and 5% CO_2. All reagents were provided by Life technologies, GIBCO BRL (Paisley, United Kingdom).

Gel electrophoresis and western blotting. Fresh samples of hippocampus were homogenized in buffer containing 100 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxicholate, 0.1% SDS, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, leupeptin and pepstatin (Sigma-Aldrich). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were carried out as described elsewhere (5), loading the same amount of protein (10-20 μg) onto the polyacrilamide gels. PVDF membranes were incubated for 2 hours with anti-adenosine A_1 receptor rabbit polyclonal antibody (PC21, 1.5 mg/ml,) and with the secondary antibody horseradish peroxidase-conjugated goat anti-rabbit Ig-G (1/50 000). Membranes were incubated in equal volumes of enhanced chemiluminescence detection solution 1 and solution 2 (Amersham Ibérica) and filters were then placed in contact with Hyperfilms ECL (Amersham Ibérica). Films were developed using a standard photographic procedure and quantitive analysis of detected bands was carried out by densitometric scanning.

Measurement of sAPP release. Eighty to 90% confluent cells, grown in 75 cm^2 culture flask, were maintained in serum-free medium for 30 minutes and then treated (or not treated, in the case of control cells) with 25, 50, 100 or 250 nM N^6-(R)-(phenylisopropyl) adenosine (R-PIA, Sigma-Aldrich company LTD, Gillingham, United Kingdom) or 160 nM TPA (Tocris, Bristol, United Kingdom) for 24 hours. In some experiments cells were pre-incubated with 5 μM bisindolylmaleimide I. hydrochloride (GF-109203X, Alexis Biochemical, San Diego, Calif) or 100 nM 1, 3-dipropyl-8-cyclopentylxanthine (DPCPX, Research Biochemicals Inc., RBI, Natick, Mass) for 25 minutes prior to the addition of R-PIA. Secreted APP was determined by western blotting of culture medium. Aliquots of medium (15 ml) were centrifuged at 4ºC for 10 minutes at 16000g. to remove non-adherent cells and cell debris. Supernantants were concentrated 15 times by centrifugation at 2000g for 40 to 60 minutes using Ultrafre-15 centrifugal filter device (Millipore, Watford, United Kingdom). A volume equivalent to an equal number of cells was loaded in each lane and SDS-PAGE electrophoresis and western blotting were performed as described above using as the primary antibody monoclonal anti-APP A4 (1/50, Sigma-Aldrich) and the secondary antibody horseradish peroxidase-conjugated goat anti mouse IgG diluted 1/8000.

Measurement of tau phosphorylation and translocation. Eighty to 90% confluent cells, grown in 25 cm^2 culture flasks, were maintained in serum-free medium for 14 hours and then treated (or not treated, in the case of control cells) with 100 nM R-PIA for 15, 60 or 120 minutes. In some experiments, cells were pre-incubated with 75 μM PD 98059 (Tocris) for 30 minutes prior to the addition of R-PIA. Cell extracts were prepared by washing cells twice with ice-cold PBS and harvesting in cold lysis buffer containing 0.5% NP-40 (Calbiochem, La Jolla, Calif), 150 mM NaCl, 10 mM Tris-Cl pH 7.4, one mM EDTA, one mM EGTA, 50 mM NaF, one mM Na_3VO_4 and protease inhibitor cocktail (Sigma-Aldrich)

diluted 1/100. Cells were centrifuged at 16 000g at 4ºC for 10 minutes to obtain cytosolic and particulate fractions. The presence of tau in these fractions was determined by SDS-PAGE electrophoresis and western blotting as described above, using as the primary antibody monoclonal anti-tau (Sigma-Aldrich) diluted 1/1000 or polyclonal anti-tau phosphorylated at $Ser^{199/202}$ (Calbiochem) diluted 1/750 and the horseradish peroxidase-conjugated secondary antibodies goat anti mouse IgG (1/3000) or goat anti rabbit IgG (1/40000).

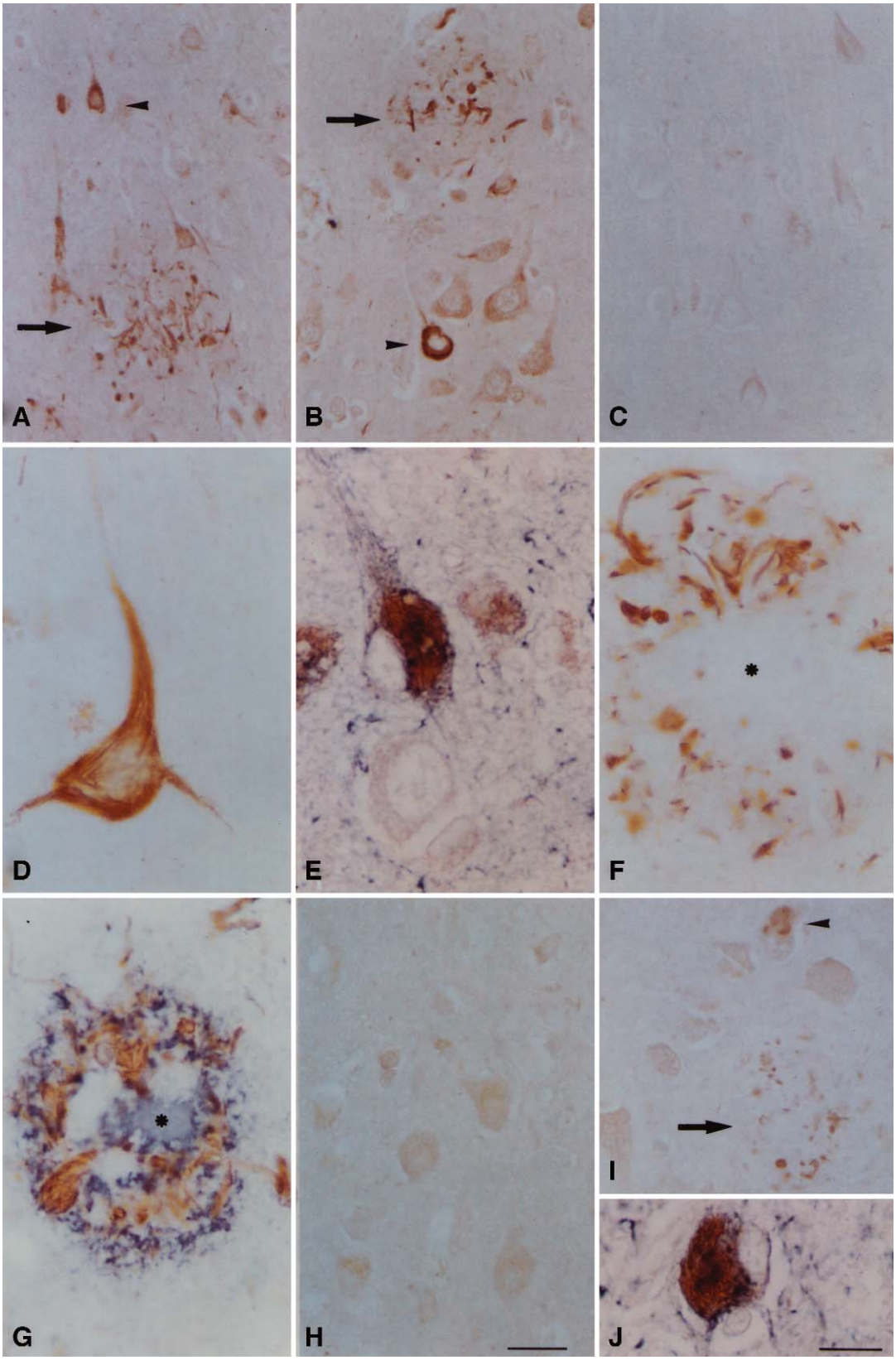
Protein determination. Protein concentration was determined by the BCA Protein Assay (Pierce, USA), following the instructions of the supplier

p21 Ras activation assay. Human neuroblastoma SH-SY5Y cells were cultured in 10-cm plates until 80 to 90% confluence and serum deprived for 16 hours. Cells were stimulated with 100nM PIA for 2 or 7 min at 37º C prior to being washed with ice-cold PBS and lysed with 50 mM Tris-HCl pH 7.5, 15 mM NaCl, 20 mM $MgCl_2$, 5 mM EGTA, 100 μM phenyl-methylsulfonyl fluoride, one mg/ml of leupeptin, one mM pepstatin A, 1% Triton X-100, and 1% N-octylglucoside, for 15 minutes at 4ºC. Insoluble material was removed by centrifugation at 12000g for 10 minutes and a pull-down assay to measure the degree of activation of p21 Ras, a procedure described elsewhere, was used (8, 13, 18).

RNA isolation, reverse transcription and SYBR Green semi-quantitative RT-PCR. Total RNA was isolated using ULTRASPEC™ reagent following the manufacturer's instructions (Biotecx, Houston, Tex). A typical yield was about 20 μg of total RNA from 100 mg of tissue. Synthesis of cDNA was performed using 2 μg of total RNA in 100 μl of a mixture of 10X TaqMan RT buffer, 0.5 mM dNTP, 2.5 μM random hexamers, 125 U MultiScribe™ Reverse Transcriptase (all reagents from Applied Biosystems) and 150 U RnaseOUT (Gibco). All reagents were mixed and incubated for 10 minutes at 25ºC, followed by 30 minutes at 48ºC and then for 5 minutes at 95ºC. Each 50 μl SYBR Green reaction consisted of 5 μl cDNA, 25 μl 2X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, Calif) and 3 μl of 300 nM forward and reverse primers (forward 5′-ATTGC TGTGG ACCGC TACCT-3′ and reverse 5′-CATCT TGTAC CGGAG AGGGA TCT-3′ for adenosine A1 receptor and forward 5′-CAGGC ACCAG GGCGT GAT-3′ and reverse 5′-CGCCC ACATA GGAAT CCTTC T-3′ for β-Actin). Quantitative PCR was performed on an ABI 7700 PCR Instrument (Applied Biosystems) by using the program parameters provided by the manufacturer: 10 minutes at 95ºC, and then 40 cycles of 15 seconds at 95ºC, and one minute at 60ºC. Optimization was performed for each gene-specific primer prior to the experiment to confirm that 300 nM primer concentrations did not produce non-specific primer-dimer amplification signal in any template control wells. Primer sequences were designed using Primer Express Software (Applied Biosystems). Specificity of the amplification product was confirmed by examination of dissociation reaction plots. A distinct single peak indicated that single DNA sequence was amplified during PCR. Each sample was tested in duplicate with quantitative PCR. Gene expression was measured by quantitation of the cDNA converted from an mRNA for a given gene relative to a calibrator sample. cDNA from unaffected human hippocampus was considered the calibrator sample. The quantitations were normalised to the β-actin gene whose expression was supposed not to vary. The relative quantitation value is expressed as $2^{\Delta\Delta C_T}$ following the instructions of the supplier.

Results

Immunoreactivity and mRNA quantitation of A_1R in necropsies from Alzheimer patients. The study of A_1R immunoreactivity was performed in one of the areas most affected by the disease, the hippocampus. In control samples taken from human brains of individuals not affected by the disease (see Methods), a weak A_1R immunoreactivity was found in pyramidal cells. This agrees with the work done by Schindler et al (36). Samples from individuals diagnosed with AD presented similar expression of A_1R in pyramidal neurons. Yet a marked increase in A_1R immunoreactivity was found in degenerating neurons with neurofibrillary tangles and in dystrophic neurites of senile plaques in the hippocampus (Figure 1A, B). The specificity of the immunostaining was tested by pre-incubation of the antibody with the corresponding antigenic peptide; the labelling was abolished under these conditions (Figure 1C). At higher magnification, A_1R immunoreactivity decorated the vast majority of neurons with neurofibrillary tangles (Figure 1D). In addition, double-labelling immunohistochemistry to A_1R and tau disclosed co-localization in neurons with tangles and in neurons with abnormal tau deposition but without tangles (pre-tangle neurons) (Figure 1E). Dystrophic neurites of senile plaques were clearly stained with A_1R antibodies (Figure 1F). Double-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labelling immunohistochemistry to A_1R and βA4 amyloid showed co-localization of A_1R immunoreactivity and βA4 amyloid in senile plaques (Figure 1G). Similar findings were observed in the frontal cortex; weak A_1R immunoreactivity was found in cortical neurons in control brains (Figure 1H). However, increased A_1R immunoreactivity occurred in neurons with tau pathology (Figure 1I, J) and in dystrophic neurites of senile plaques (Figure 1I).

To learn whether the distinctive pattern of expression of A_1R in samples from AD was unique or not, the expression of another subtype of adenosine receptor, $A_{2A}R$, and of another member of the superfamily of G protein coupled receptors, the group I metabotropic glutamate receptor, were assayed in similar samples. $A_{2A}R$ is strongly expressed in striatum and, with much less intensity, in other brain areas. In fact, there is no specific label in hippocampal samples taken from unaffected individuals (not shown). In contrast, in samples from Alzheimer patients, $A_{2A}R$ immunoreactivity was found in glial cells (Figure 2A, B). $A_{2A}R$ positive cells (Figure 2A) were identified as microglia by double-labelling immunohistochemistry using anti-$A_{2A}R$ antibodies and lectin from *Lycopersicum esculentum* as a specific marker for microglia (Figure 2B). The pattern of distribution of group one mGluR was similar in hippocampal samples from non-diseased individuals (Figure 2C) and from Alzheimer patients (Figure 2D). Similarly, no differences were seen in the frontal cortex regarding the localization and distribution of group one mGluR expression (Figure 2E, F).

To learn whether the differing pattern of expression of A_1R in control samples and samples from Alzheimer-affected brains correlated with differences in overall expression of the receptor in hippocampus, immunoblots of homogenized tissue from controls and patients were obtained. It was found that the amount of protein receptor is slightly higher in degenerated hippocampus (Figure 3); this is more evident for the high molecular weight form corresponding to the glycosylated mature receptor. To discover whether this slight increase in protein is due to an increase in the transcription of the gene, a quantitative PCR approach was used (see Methods). The results of mRNA expression indicate that no statistically significant changes in mRNA expression occur in AD (Table 1). These results suggest that transcription of A_1R gene is not altered in AD but also that there is slowing down in the degradation of the protein, together with a change in the histochemical distribution of the receptor.

	$\Delta\Delta Ct$	$2^{\Delta\Delta Ct}$
AD1-C1	-0.69	0.620
AD2-C1	-1.02	0.493
AD1-C2	0.03	1.021
AD2-C2	-0.29	0.818

Table 1. *A_1R mRNA expression by quantitative PCR analysis.* Quantitative PCR analysis was performed using human hippocampus mRNA from two unaffected (C1 and C2) and two AD affected (AD1 and AD2) brains.
C_T: Threshold cycle at which there is a statistically significant increase in ΔR_n (ratio between emission intensity of SYBR Green and the emission intensity of the Passive Reference)
ΔC_T: difference between the mean C_T values of the A_1R and endogenous control β-actin amplifications.
$\Delta\Delta C_T$: difference between the mean ΔC_T of A_1R samples in AD cases and the mean ΔC_T values in control cases.
$2^{-\Delta\Delta CT}$: quantitation of A_1R in AD cases relative to control cases.

Role of A_1R in APP processing and tau distribution. To investigate the putative role of A_1R in degenerative brain structures, a human neural cellular model naturally expressing the receptor (neuroblastoma SH-SY5Y cells) was used for signalling, tau phosphorylation and precursor amyloid protein (APP) processing assays. First, the effect of activation of A_1R upon production of soluble APP was tested. As indicated in Figure 4, the specific agonist of the receptor, R-PIA, was able to produce a dose-dependent increase in the level of the soluble glycosylated forms of amyloid precursor protein secreted to the medium. As a positive control, an activator of protein kinase C (TPA) was used. To confirm that this effect upon production of soluble APP was receptor-

Figure 1. (Opposing page) *A_1 adenosine receptor immunoreactivity in hippocampus and frontal cortex.* A_1 receptor immunolabelling in hippocampus from AD affected brains in the absence (**A**, **B**, **D**, **F**) or presence (**C**) of the peptide which binds to the anti-adenosine A_1 receptor antibody. Double labelling immunohistochemistry of: (**E**) A_1 receptor (brown precipitate) and tau (dark blue precipitate), (**G**) A_1 receptor (brown precipitate) and β-A4 peptide (dark blue precipitate). A_1 receptor immunostaining in frontal cortex from brains unaffected (**H**) and AD affected (**I**). Double-labelling immunohistochemistry of A_1 receptor (brown precipitates) and tau (dark blue precipitates) in frontal cortex from AD affected brains (**J**).
Panels **A**, **B** and **I**: Arrowhead indicates neurofibrillary degenerated neurons and arrow indicates dystrophic neurites of senile plaques.
Panel **F** and **G**: Asterisk indicates amyloid core.
A-C, **F-I** bar in **H** = 25 μm; **D**, **E**, **J**, bar in **J** = 10 μm.

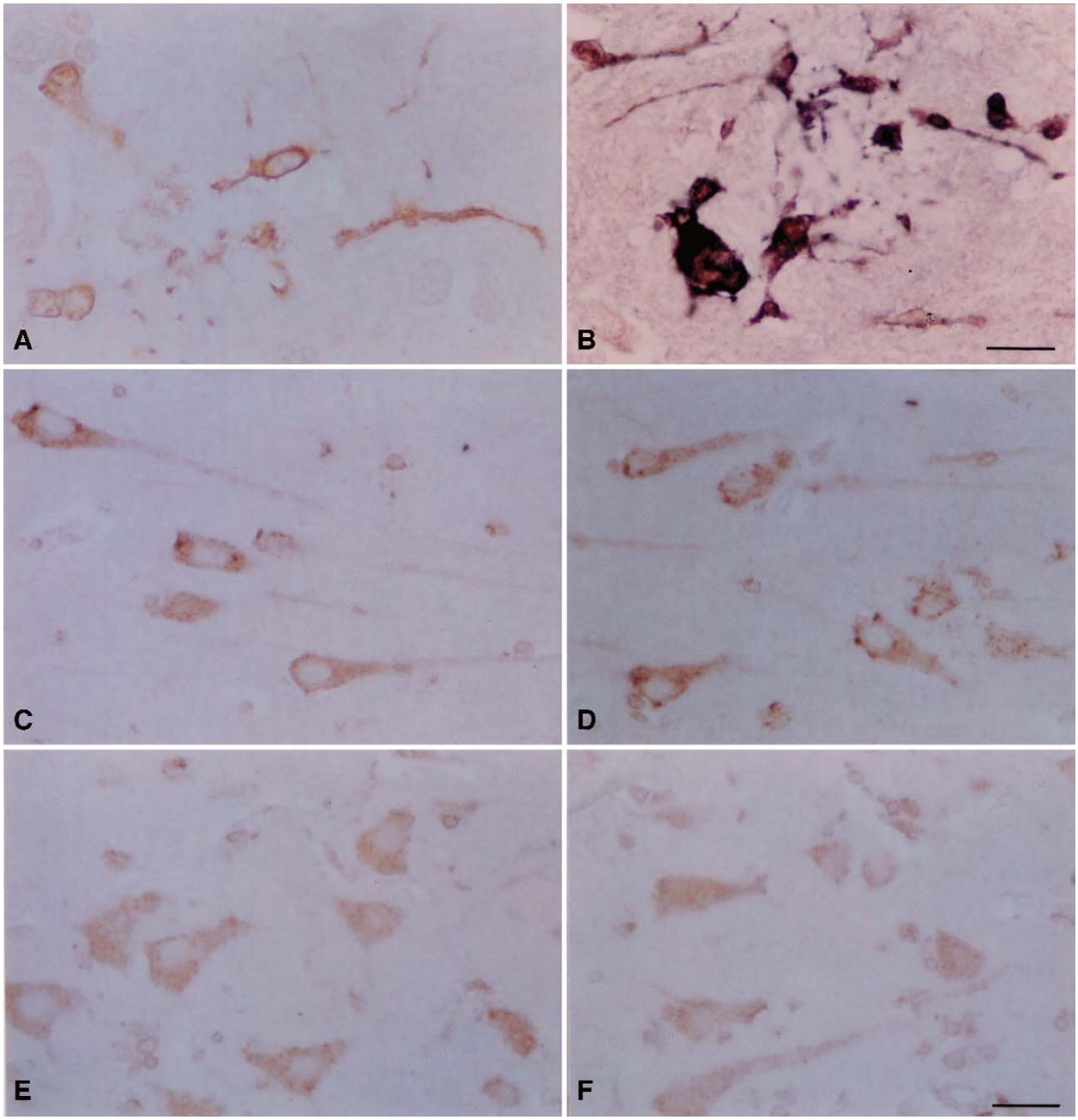

Figure 2. *Adenosine A_{2A} receptor and metabotropic glutamate receptor immunoreactivity.* Adenosine A_{2A} receptor immunolabelling in hippocampus from AD affected brains (**A**). Double-labelling of A_{2A} receptor (brown precipitate) and microglia-specific carbohydrates (dark blue precipitate). (**B**). Metabotropic glutamate receptor type I immunoreactivity in hippocampus (**C**, **D**) and frontal cortex (**E**, **F**) from unaffected (**C**, **E**) and AD affected brains (**D**, **F**).
A, **B**, bar in **B** = 10 μm; **C**, **D**, **E**, **F**, bar in **F** = 25 μm.

mediated, the specific antagonist DPCPX was used. This reagent, which was ineffective when used alone, completely prevented the effect of the A_1 specific agonist. Interestingly, the effect was mediated by activation of protein kinase C (PKC), since it was blocked by the PKC inhibitor bisindolylmaleimide (GF-109203X). This strongly indicates that the enzyme that processes APP when A_1R is activated is α-secretase which, in

contrast to β-secretase, is PKC sensitive (21, 23). The effect of R-PIA on p21 Ras activation was also investigated (see Methods). Activation of A_1R led to a rapid activation of this GTP binding protein that was already evident at 2 minutes of treatment with R-PIA (Figure 5). In line with the role of p21 Ras upstream of the MAPK cascade, R-PIA led to a quick transient increase in ERK1 and ERK2 phosphorylation (Figure 5). To demonstrate the specificity of the effect, an antagonist of the receptor, DPCPX, completely blocked the action of the agonist R-PIA upon phosphorylation of MAP kinases (data not shown). The consequences of A_1R activation on tau distribution and phosphorylation were assayed. For this purpose cells were treated with R-PIA and cell extracts were processed as described in Methods to separate cytosolic and particulate fractions. As early as 15 minutes, treatment with R-PIA led to a decrease in the total tau present in the nonparticulate fraction. This decrease correlates with the increase of the protein in particulate fractions where tubulin is present (Figure 6). This R-PIA-induced translocation of tau from cytosol to particulate fractions was prevented by pretreatment with the ERK kinase inhibitor PD-98059, thus indicating that translocation correlates with ERK phosphorylation. Furthermore the degree of phosphorylation of tau present in the particulate fraction was higher in samples obtained from cells treated with the agonist than those obtained from naive cells. Since A_1R is negatively coupled to adenylate cyclase, it is therefore shown in this human cell model that adenosine, via A_1R, activates a cAMP-independent

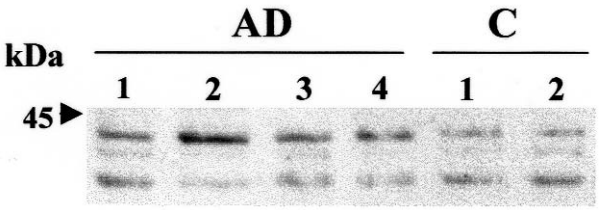

Figure 3. *A_1R expression in human hippocampus.* Protein expression of A_1R in hippocampal samples of 4 Alzheimer affected brains (AD) and 2 unaffected brains (C) was determined by SDS-PAGE and western-blotting using the specific antibody against A_1R (see Methods).

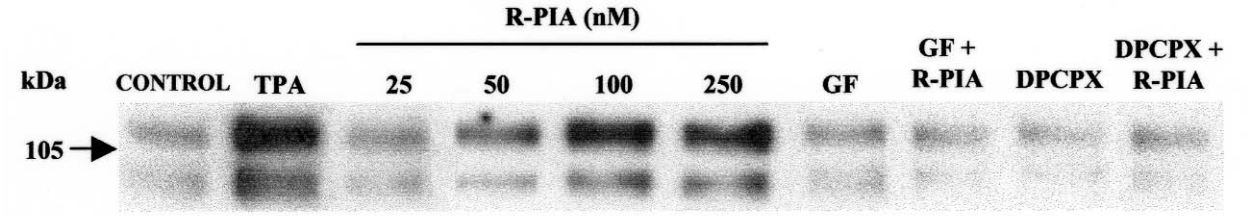

Figure 4. *A_1R agonist-mediated APP secretion.* SH-SY5Y neuroblastoma cells were serum deprived for 30 minutes and then stimulated with 25, 50, 100 or 250 nM R-PIA for 24 hours. In some experiments cells were preincubated with 5 μM GF-109203X (GF) or 100 nM DPCPX prior the addition of 25 nM R-PIA. TPA (160nM) stimulation was used as positive control. Secreted soluble APP was determined by western blotting of the concentrated culture media as described in Methods. A representative immunoblot from 5 separate experiments is shown.

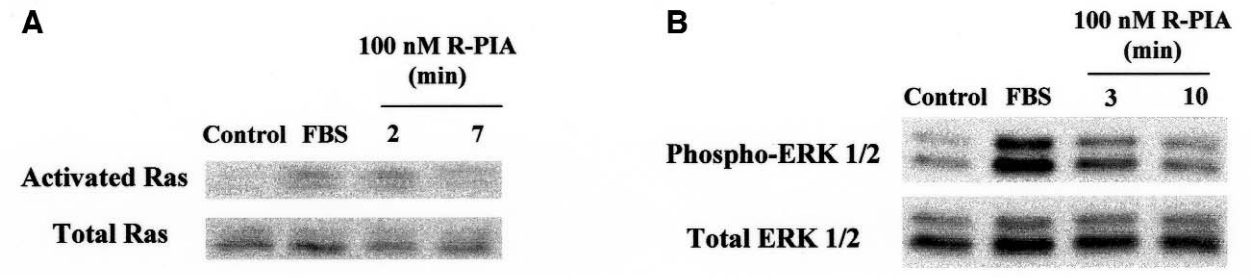

Figure 5. *R-PIA-mediated p21^{ras} activation and ERK1/2 phosphorylation.* Serum starved cells were stimulated with 100 nM R-PIA for the indicated time or with FBS as positive control. Cells were lysed as indicated in Methods. (**A**) Lysates were incubated with GST-RBD fusion protein (see Methods), and activated, and total p21 Ras were detected by immunoblotting (see Methods). (**B**) The presence of phosphorylated ERKs (Phopho-ERK1/2) and the total amount of ERK1/2 (Total ERK1/2) in lysates were determined by immunoblotting (see Methods). Representative Western blots of 4 experiments are shown.

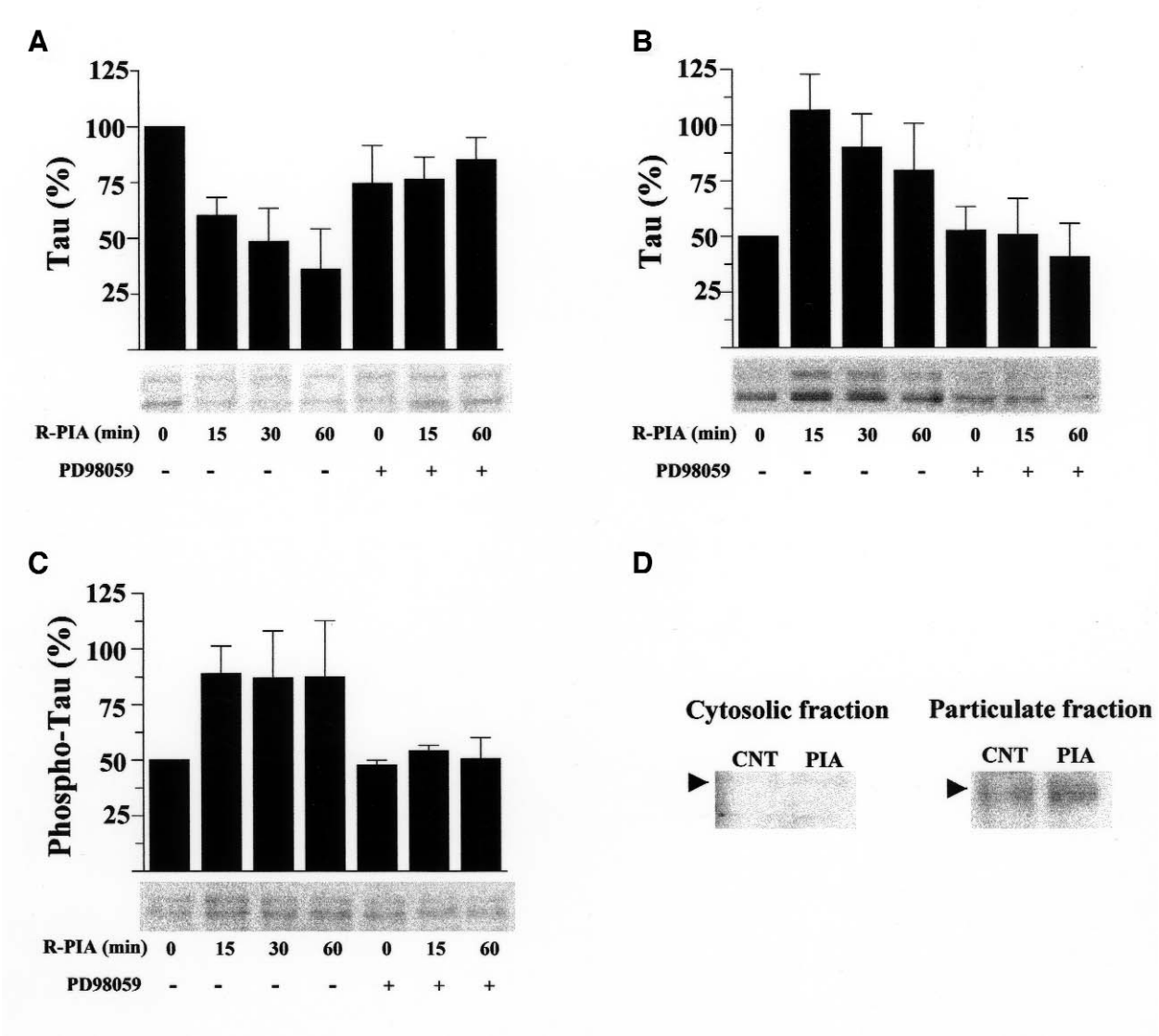

Figure 6. *R-PIA-mediated tau translocation and phosphorylation.* Serum starved SH-SY5Y cells were stimulated with 100 nM R-PIA for 15, 30 or 60 minutes in the absence (-) or presence (+) of 75 μM PD98059. Cytosolic and particulate fractions from lysed cells were obtained as indicated in Methods. Total tau in the cytosolic fraction (**A**) or in the particulate fraction (**B**) was determined by immunoblotting using a monoclonal anti-tau antibody. Phosphorylated tau in the particulate fraction (**C**) was determined by immunoblotting using a polyclonal anti-phosphorylated (Ser199/202) tau antibody. The intensity of the immunoreactive bands (upper band in the blots shown in **A**, **B** and **C**) on X-ray film was measured by densitometric scanning. All values were normalised to non-treated cells and expressed as percentages. The results are expressed as means ±SEM from 3 independent experiments. (**D**) Tubulin immunoreactivity in cytosolic and particulate fraction in non-treated cells (CNT) and in cells stimulated with 100 nM R-PIA for 30 minutes.

and p21ras-dependent cascade which leads to MAP kinase activation. This induces the tau phosphorylation necessary for tau traslocation from the cytoplasm to the cytoskeleton These results in SH-SY5Y cells also indicate that adenosine, through A_1Rs, regulates a variety of processes that are reported to be involved in the pathophysiology of AD.

Discussion

The results presented in this paper indicate that at the protein level A_1Rs are slightly increased in the hippocampus from Alzheimer brain. In contrast, the level of transcription of the gene, measured by quantitative PCR, is similar in control and Alzheimer brain. In a careful study performed using DNA arrays probed with

mRNA from frontal cortex, it has been found that the level of specific mRNA for A_1R is similar in control and Alzheimer brain; the difference between samples was in no cases greater than double or less than half (results in preparation). Schindler et al (36) have recently reported a mapping of the distribution of A1R in various regions of healthy human brain performed by immunohistochemistry. It should be noted that the antibodies used by Schindler et al (36) and by us were raised against different epitopes of the protein.

In this report, which is the first describing a careful study of immunolocalization of A_1R in necropsies from AD patients, it is clearly shown that redistribution of these receptors occurs in neurons with abnormal tau deposition (ie, neurons with neurofibrillary tangles and neurons with pre-tangles, as seen with double-labelling immunohistochemistry) and in dystrophic neurites of senile plaques. Accumulation of A_1R in neurodegenerative structures in hippocampus from patients is, in our opinion, a relevant finding that does not happen in the case of group I metabotropic glutamate receptors. In fact, group I metabotropic receptors appear mainly in pyramidal neurons irrespective of the type of region, hippocampus or frontal cortex, and irrespective of whether the samples are from controls or from patients. It is also noteworthy that the A_{2A} adenosine receptor subtype, which is hardly expressed in hippocampus, appears in glial cells in samples taken from AD patients. As indicated by the double labelling with lectin from *Lycopersicum esculentum*, the A_{2A} receptor is expressed in microglia present in affected hippocampus. Therefore the location of A_1Rs in neurodegenerative structures and of $A_{2A}R$ in microglia from patient hippocampus indicate that adenosine can play a role in regulating the events taking place along the development of AD.

It was not known whether there is any role for adenosine receptors in processes involved in the pathophysiology of AD. At a molecular level one of the characteristics of AD is the abnormal processing of APP and the occurrence of hyperphosphorylated tau in tangles (for review, see 20, 32). Although the mechanisms for APP processing are well known, it is unclear what the factors are that regulate this process in brain from healthy and from Alzheimer patients (7, 19, 38, 42). In this paper the participation of A_1R in APP processing in a human neuronal cell model is described. In fact, activation of A_1R in human neuroblastoma SH-SY5Y cells led to the production of the soluble secreted form of APP. Since the adenosine-mediated event is blocked by an inhibitor of protein kinase C, the cleavage of the protein is probably performed by α-secretase, thus giving rise to the secretion of non-amyloidogenic forms of APP (23). Although the mechanisms of redistribution of A_1R in dystrophic neurites of senile plaques are not known, this redistribution may be related to the pathological state of APP metabolism in AD, possibly enhancing the conversion of APP into sAPP and preventing further deposition of β-amyloid. Redistribution of A_1R in amyloidogenic plaques may thus be involved in compensatory mechanisms occurring in developing AD.

Hyperphosphorylation of tau is required for tangle formation; this event prevents binding of tau to components of the cytoskeleton and favours polymerization and fiber formation (2, 30). Although the domains of tau that participate in the interaction with cytoslekelton have consensus sites for phosphorylation (2), it is not known whether phosphorylation is really necessary for the binding of tau to the cytoskeleton. In this paper an involvement of A_1R in tau phosphorylation and its translocation towards cytoskeleton is demonstrated. As shown in Figure 6, A_1Rs mediate tau phosphorylation by a mechanism blocked by inhibitors of MAP kinases and promotes translocation of the protein toward particulate fractions. In these particulate fractions tau is probably bound to cytoskeletal proteins since tubulin, which can associate to tau, is also present in the insoluble but not the soluble fraction of the cell extracts (Figure 6). Both phosphorylation and translocation are blocked by the inhibitor of MAP kinases, thus indicating that ERKs have a key role in tau phosphorylation and translocation mediated by A_1R activation. Assuming that these signalling pathways also occur in neurons present in human brain, their operation would be relevant due to the precise location of A_1R in neurofibrillar tangles, which again suggests that adenosine may play a role in regulating the progression of AD. Strong phosphorylated (active) MAPK/ERK immunoreactivity co-localizes with early abnormal tau deposition in neurons and glial cells in AD and other taupathies. Furthermore, active Ras is also increased in AD, thus suggesting activation of the Ras/MAPK cascade associated with tau phosphorylation in AD (12). From results by Mandelkow et al (31) and Chin et al (4), it is known that tau bound to cytoskeleton seems to be less susceptible to forming neurodegenerative structures such as the paired helical fragments found in neurofibrillar tangles. These results indicate that by releasing nonamyloidogenic forms of APP and by promoting ERK-mediated tau phosphory-

lation and translocation, adenosine would be slowing down the progression of the disease. Taken together, these data indicate that adenosine receptors (A1R and A2AR) can be considered potential targets for therapeutic intervention in AD.

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